Endogenous Cardioprotective Agents: Role in Pre and Postconditioning.

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Abstract

Cardiovascular diseases (CVD) are the leading cause of death and disability in the occidental countries. The major pathological consequences of CVD derive from the harmful effects of acute myocardial ischemia and following reperfusion injury. Protection of the myocardium against damaging ischemia/reperfusion injury is made possible by "conditioning protocols". Conditioning is obtained applying a few periods of brief ischemia and reperfusion in the event of prolonged (index) ischemia that may cause myocardial infarction. Whilst the conditioning stimulus is applied *before* the index ischemia in ischemic *pre-conditioning*, it is applied *after* the event in *post-conditioning*. Pre and post-conditioning stimuli can be applied in a different/remote organ (*remote pre- and post-conditioning*); in this case conditioning stimulus can also be applied *during* the index event, in the so called *remote per-conditioning*. All these endogenous cardioprotective strategies recruit endogenous cytoprotective agents and factors that elicit specific cardioprotective pathways. Here, we provide an overview of many of these cardioprotective factors with respect to the literature and underline their major features and signaling mechanisms. Enphasis is given to endogenous cardioprotective agents acting or not on surface receptors, including chromogranin A derivatives, ghrelin-associated peptides, growth factors and cytokines, and to microvesicles and exosomes. Moreover the cardioprotective effects of gasotransmitters nitric oxide, hydrogen sulphide and carbon monoxide are reviewed. The possible clinical translation of these knowledge for future successful therapies is briefly and critically discussed.

Key words: Cardioprotection; Chromogranin A derivatives; Exosomes; Ghrelin-associated peptides; GPCR activators; Growth factors; Ischemia/Reperfusion; Tyrosine kinase receptor activators.

Introduction

Cardiovascular disease (CVD) is the leading cause of death and, in particular, acute myocardial infarction (AMI) remains a major cause of mortality and morbidity worldwide. The restoration of blood and oxygen to the ischemic myocardium is the only way to limit ischemia injury, but reperfusion paradoxically exacerbates the cellular damage due to ischemic insult. In recent years it has become clear that the myocardial ischemia/reperfusion (I/R) injury can be reduced by protective strategy of ischemic conditioning, which stimulated considerable research into the mechanisms of cardioprotection.

Cardiac 'conditioning'

Myocardial conditioning refers to the stimulation of intrinsic cardioprotective mechanisms by short periods of non-lethal ischemia that target lethal tissue injury caused by a longer period of ischemia (the so called *index ischemia*). The conditioning stimulus can be applied before (Preconditioning, PreC), during (Perconditioning, PerC), or immediately after (Postconditioning, PostC) the '*index' ischemia*.

The *gold standard* treatment against I/R injury by which other therapies are judged is the *ischemic PreC*. It was described in 1986 by Murry and colleagues [1] and consists of a series of brief periods of I/R (a few minutes) performed before the infarcting ischemia (it is also called *mechanical PreC*). This study has shown that infarct size can be modified by triggering endogenous mechanisms of protection. PreC not only reduces all form of cell death, but also endothelial dysfunction and activation as well as neutrophil adhesion and inflammatory response. It also reduces stunning (the contractile depression which follows ischemia) and arrhythmias [2,3]. Moreover, PreC ameliorates vascular responsiveness to endothelial-dependent vasodilatation and can slow mitochondrial metabolism [2-4].

It has been shown that PreC can also be induced by exercise protocols [5]. Actually, the cardioprotection of PreC induced by mechanical manoeuvres (*i.e.* brief cycles of I/R) or exercise triggers the release of various molecules (*e.g.* adenosine, acetylcholine, opiates, bradykinin and possibly other factors treated in this review) which are responsible for the triggering of intracellular cardioprotective pathways. A similar cardioprotection can be also induced directly by the infusion of one of these endogenous agonists or other agents for few minutes; that is *pharmacological PreC* [6]. Importantly, protection by either ischemic, pharmacological or exercise PreC has been demonstrated in several animal species, including humans, and can be abolished by antagonists of these endogenous agents [7].

However, PreC can be useful in programmed cardio-surgery interventions. It is not useful in the patient with AMI because it must be performed prior to ischemia. Later, ischemic/mechanical PostC, which involves to subject the myocardium to cycles of brief reperfusion and ischemia (a few seconds) at the onset of reperfusion, has also shown to be protective against I/R injury [8-10]. PostC reduces: infarct size, apoptosis, post-ischemic arrhythmias, endothelial-dysfunction and -activation. However, it is not clear if it can reduce myocardial stunning [11,12]. Cardioprotection by

pharmacological PostC has also been described. It is obtained with the infusion of some of the agents able to induce PreC, but these agents must be applied very soon in reperfusion, because the first minutes of reperfusion are critically important [13].

More recently, ***remote*** *ischemic PreC, PerC and PostC* have also proved to be protective against I/R injury [14-19]. These procedures consist in subjecting a limb or organ, remote from the heart, to brief cycles (a few minutes) of ischemia and reperfusion, *prior to or during cardiac ischemia* and *after reperfusion*, respectively.

The above described ischemic conditioning protocols are potent cardioprotective interventions that decrease infarct size after relatively long periods of cardiac ischemia followed by reperfusion. In fact, though early reperfusion in AMI limits infarct size, there may still be significant myocardial cell death due to the pathophysiological mechanisms triggered by reperfusion itself, which is termed lethal reperfusion injury. Reperfusion injury occurs as a result of a number of pathophysiological mechanisms that are triggered at the time of oxygen reintroduction after an ischemic period. Before to consider the endogenous factors responsible of cardioprotection we briefly describe the general mechanism of reperfusion injury and protection.

Mechanisms underlying reperfusion injury

The most important consequence of reperfusion injury is an increase in the open probability of the *mitochondrial permeability transition pore* (mPTP). The opening of mPTPs leads to the loss of mitochondrial inner membrane potential, which in turn triggers various modes of cell death including necrosis, apoptosis and necro-apoptosis [20-22]. Alteration of two or more physiological mechanisms, including cellular pH, calcium homeostasis and production of reactive oxygen and nitrogen species (ROS/RNS), are responsible for the pore opening.

Intracellular calcium homeostasis becomes dysfunctional during ischemia *via* injury to the sarcolemmal membrane and the sarcoplasmic reticulum, leading to an increase in cytosolic calcium. This calcium overload is further accentuated at the time of reperfusion by calcium influx through reverse mode Na^+-Ca^{2+} exchange [23,24], and besides to promote mitochondrial calcium overload and mPTP opening (favored by pH recovery due to Na^+/H^+-exchanger activity) has several other deleterious effects, including formation of contraction band necrosis [24].

Also *ROS/RNS* production is altered by ischemia and subsequently accentuated at the time of reperfusion and contribute to cause injury to the myocardium as well as to the endothelium [25]. Ischemia also triggers inflammation leading to an increase in vascular permeability in the region of the infarct, readily permitting neutrophil migration into the ischemic risk zone upon reperfusion [26]. The release of ROS and toxic substances by activated neutrophils exacerbates tissue damage induced by reperfusion [27,28].

Another important pathological mechanism contributing to reperfusion injury is the *"no-reflow phenomenon"*. No-reflow is the lack of blood flow, which is not restored to all segments of the microvasculature in the post-ischemic

myocardium distal to site of occlusion, even after the obstruction has been treated and removed. No-reflow in the setting of patients undergoing revascularization after ST-segment-elevation AMI is a result of microvascular obstruction due to several mechanisms [29-31]. Among these are distal thromboembolism associated with revascularization of the coronary vessel and the endothelial injury occurring as a consequence of oxidative and inflammatory stress.

Protective pathways and mechanisms against lethal reperfusion injury

As previously mentioned, conditioning protocols are dependent on signaling pathways involving several factors released from ischemic tissue and acting in autocrine/paracrine fashion (Fig. 1). Many of these, with their specificity will be considered in the present review, including also PAF (platelet activating factor), TNF-α (tumor necrosis factor-alpha), S1P (sphingosine-1-phosphate) and chromogranin A derivatives (*i.e.* catestatin and vasostatin), which may be counted among endogenous triggers of pre- and/or post-conditioning. These ligands may couple to G-protein-linked receptors, or tyrosine kinase receptors, or may activate directly intracellular signaling pathways, including kinases, such as protein kinase C (PKC), nitric oxide synthase (NOS), mitochondrial ATP-sensitive potassium channels (mKATP), and may promote ROS/RNS production with signaling role. For the majority, if not for all, of the endogenous ligands the final protective target of the pathways is the mitochondria, where the signaling induces protection by preventing mPTP formation, which is considered the end-effector of the protective pathway (see below).

Regardless the fact that the ligand is formed during pre- or post-conditioning procedure, it protects against reperfusion injury, which follows the index ischemia. Similar cardioprotective mechanisms and pathways are operative in the reperfusion phase. These include acidosis, ROS signaling, as well as protective signaling pathways, namely cGMP/PKG (cyclic guanosine monophosphate/Protein kinase G), RISK (Reperfusion Injury Salvage Kinase) and SAFE (Survivor Activating Factor Enhancement).

Acidosis in the initial phase of reperfusion is protective. Its protective role has been clearly demonstrated in PostC experiments and has been attributed to the direct inhibition exerted on mitochondria, where it prevents mPTP opening [32]. Also transient pre-ischemic acidosis resulted to be protective [33]. Although PreC decreases tissue acidosis and anaerobic glycolysis during the subsequent sustained ischemic period [34], it avoids fast pH recovery at the beginning of reperfusion [24,35]. Therefore, both in pre- and post-conditioned hearts the persistence of a slight acidosis in the initial phase of reperfusion plays a crucial role in the protection against reperfusion damage.

ROS/RNS signaling in the initial phase of reperfusion is protective. ROS/RNS are double edged swords whose protective role have been clearly demonstrated in PostC experiments and have been attributed to the direct activation of kinases, including PKC [36-38]. Also transient pre-ischemic ROS/RNS formation resulted to be protective and it seems that *redox signals* arise during the reperfusion phase of the brief PreC cycles of I/R [39] or during hypoxia (not anoxia) PreC [40]. Although PreC modulates ROS/RNS production during the ischemic period [34,41], it also modulates and

limits (not avoids) ROS/RNS production during reperfusion [35,37,38,40-42]. Therefore, both in pre- and post-conditioned hearts the persistence of a slight production of ROS/RNS in the initial phase of reperfusion plays a pivotal role in the protection against reperfusion injury.

The acidosis and redox signaling in early reperfusion contribute to the activation of *cardioprotective pathways*, which include several processes and enzyme activation/inhibition. They have been described in several excellent reviews, to which the reader is kindly redirected; see for example [3-6,10,14,16,35-38,43-47]. These pathways are here briefly described when considering the single ligand in the next section of the present review. In brief, three mainstream cardioprotective pathways are described, namely the cGMP/PKG pathway, which starts from nitric oxide formation and guanylyl cyclase (GC) activation, the RISK pathway, which includes activation of protein kinase B (PKB, also known as Akt) and extracellular signal-regulated kinase (ERK)1/2, and the SAFE pathway, which requires the activation of the signal transducer and activator of transcription 3 (STAT3). These pathways converge on the mitochondria, which are considered the intrecellular effector to limit I/R injury.

*Intracellular End Effectors (Mitochondria***)**

Mitochondria are major common intracellular target structures in both PreC and PostC by the majority of the endogenous cardioprotective factors [12,37,47-49]. These organelles represent 40% of cardiomyocytes mass and are the site of aerobic ATP production and hence they are fundamental for energy production to sustain myocyte functions and survival. Mitochondria have also a role of paramount importance in apoptosis, autophagy and necrosis [12,37, 47-49]. Actually, the final effector of protection is likely the mPTP, and the cardioprotective signaling pathways are effective by preventing pore formation. Various protein kinases collaborate to activate the mKATP channels, thereby leading to a modest production of ROS [37,39,45]. Kinases and ROS-signaling contribute to inhibit the opening of the mPTP [7,23,35,37,48,49]. Also nitros(yl)ation of mitochondrial membrane proteins seems causally involved in cardioprotection by PreC and PostC [38,49-52]. Several agents act on mitochondria, including adenosine. In fact, a mitochondrial localization of adenosine A_{2B} receptor has been recently reported in cardiomyocytes, which exerts a cardioprotective role [53]. Opening of mKATP channels depends on cytosolic PKG and involves PKCε in the mitochondria, which operates the transmission of the signal from PKG to the mKATP [4,12,44,45]. It has also been suggested that mitochondrial PKG may be contained in the socalled *signalosomes:* discrete multimolecular complexes containing critical signaling factors, formed starting from sarcolemmal caveolae, which reach the mitochondria traveling in defined cytoplasmic systems [54]. Mitochondrial connexin 43 (CX43), located on the inner membrane, appears to be fundamental in these processes. Downregulation of CX43 mitigates both mKATP-dependent cardioprotection and ROS production [55]. Actually, the cardioprotection by the mKATP opener, diazoxide, depends on phosphorylation of CX43 by PKC [56]. This suggests some sort of reverberant signaling. In fact, PKC phosphorylates

CX43, leading to ROS production, but ROS may cause PKC activation. Moreover very recently CX43 nitros(yl)ation has been proposed as an important modification in the regulation of mitochondrial function [57]. Nevertheless, the role (channel, scaffold or signaling molecule) exerted by mitochondrial CX43 in the protective processes is not well known yet. Interestingly, PostC with diazoxide may also promote nitros(yl)ation of several mitochondrial proteins, including putative components of mPTP [38].

Although STAT3 are transcription factors, their effects in I/R are too fast to act as gene modulator. Actually, phosphorylated STAT3 translocates to mitochondria to modulate electron transport [4,12,37,58]. It also phosphorylates and, therefore, inactivates GSK3β, a downstream target and point of convergence of RISK and SAFE pathways [58-60]. Phosphorylated STAT3 could inhibit mPTP formation. Indeed, PostC increases phosphorylation of mitochondrial STAT3 in pigs, thus improving calcium retention capacity and complex I respiratory function [61]. Upstream to STAT3 there is JAK. In fact, its inhibition avoids both mitochondrial STAT3 phosphorylation and PostC cardioprotective effect. Moreover, mitochondrial STAT3 co-immunoprecipitates with cyclophilin D, the putative target of cyclosporine A (CsA), a mPTP desensitizer [62]. Cyclosporine A inhibiting mPTP opening limits infarct size in several animal species, including mice [62] and pigs [63-64], but not in rats [65]. Also in AMI patients during primary percutaneous coronary intervention, CsA given as an intravenous bolus just before reperfusion limited infarct size, as suggested by analysis of creatine kinase release [66]. Similarly, cardioprotection by CsA was also reported for patients subjected to coronary artery bypass surgery [67]. However, because of the adverse effects and non-selectivity of CsA for the mPTP, more specific and safer novel mPTP inhibitors must be identified and developed in order to implement mPTP inhibition as a therapeutic cardioprotective strategy. Nevertheless, despite intensive investigation, the actual molecular identity of the pore component(s) of the mPTP remains unknown, thereby making difficult the research for appropriate drug(s) to target this pore.

Below we consider the endogenous cardioprotective factors, with their specificity in inducing the above described general mechanisms of protection.

Cardioprotective Agents Acting on Surface receptors

G protein-coupled receptors (GPCRs) activators

Adenosine

Adenosine is the first factors involved in PreC. It is a purine nucleoside generated both in intracellular and extracellular compartments of the myocardium. Adenosine levels rise following to various pathophysiological stimuli, in response to moderate alteration of energy state and to diverse factors (adrenaline, NO, histamine) and signaling pathways. In

particular, adenosine is a part of a metabolic signal, released after energetic discrepancies or perturbations in the equilibrium between myocardial O_2 supply and demand. By binding to adenosine receptors (ARs) 1, 2A, 2B, 3 (A_1AR, A_{2A}AR, A_{2B}AR, A_3AR), adenosine improving the cellular energy balance modulates several aspect of cardiovascular function, including autonomic control of the heart, cardiac and vascular resistance to insults, cardiovascular growth and remodeling, conduction of the cardiac impulse, coronary perfusion, cronotropy and inotropy [68,69].

Adenosine has been extensively studied as a factor of cardioprotection in the I/R injury. The first step in the generation of adenosine is the conversion of ATP/ADP to AMP, carried out by the ectonucleoside triphosphate diphosphohydrolase CD39, an enzyme induced by hypoxic or ischemic conditions [70]. Indeed, $cd39^{-/-}$ mice show a blunted increase of adenosine after myocardial ischemia [71]. The second step is the conversion of AMP to adenosine, catalyzed by the ecto-5'-nucleotidase CD73, an enzyme induced by inflammation, ischemia and hypoxia [70]. Adenosine generated during or after an ischemic insult signals through ARs is able to protect the myocardium and limit cellular injury, and is involved in pre- and PostC [69,72]. The I/R damage involves several factors, such as inflammation and innate immunity, oxidative stress, ionic and energetic disturbs. The adenosine-AR system can target all these determinants of the myocardial injury and repair [69]. Indeed, ARs are GPCRs that can activate pro-survival RISK signaling, with involvement of ERK1/2, phosphatidylinositol 3-kinase (PI3K)/Akt, PKC and downstream mKATP channels [35,43,44,69]. An important role for EGFR transactivation, *via* a MMP-dependent mechanism, in AR cardiac protection has also been postulated [73], as well as maintenance of mitochondrial function and inhibition of mPTP [35,43,44,69]. Interestingly, in A_1R protection the beneficial modulation of autophagy has also been implicated [74], while the inflammatory process that takes place during the ischemia-reperfusion process may be sensitive to A_2AR and A_3AR [75,76] and not to A_1, which more directly protects the myocardium when activated before ischemia, but not in reperfusion [69,73]. A_{2B}AR cardioprotection has been linked to glycogen synthase kinase 3 beta (GSK3β) and the mPTP [77], and recently to the stabilization of the circadian rhythm protein period 2(Per2) with following metabolic adaptations [69,70]. Also, adenosine binding on A_{2B}AR results in inhibition of mitochondrial ROS generation through ERK1/2, PI3K and NOS activation [78] and in modulation of TNFα and neutrophils inflammation [79]. The inflammation processes in the post-ischemic phase of reperfusion are also target of A_3AR [80]. Interestingly, A_3AR also targets the pre-ischemic and ischemic phase [81-83]. Thus, while A_1 and A_3AR participate in cardioprotection in the ischemic phase, A_{2A} and A_{2B}AR involvement is important in reperfusion. Therefore, A_1/A_3AR can be envisioned as crucial in PreC, with A_{2A}/A_{2B}AR crucial in ischemic PostC [84-87]. Such phenomenon is more relevant to clinical scenario of AMI, since it is feasible in the clinical setting of reperfusion. Moreover A_1, A_2 and A_3AR knockout mice could be protected by PreC, whereas A_{2B}AR knockout mouse could not be conditioned. Yet, there is evidence suggesting a positive interaction between A_{2A} and A_{2B}ARs in some protocol of cardioprotection [77,88]. The current

thought is that all the ARs are required and interact to produce cardioprotection: activation of A_1 or A_{2A}AR modulates A_{2B}AR functionality [89-91]. Such interactivity is an actual field of study and controversy in the pharmacological modulation of cardioprotection, since the main clinical studies (AMISTAD I and II) [92,93] showed some beneficial effect of adenosine only in anterior AMI, with high dose adenosine in an early reperfusion protocol.

Adipocytokines

Adipose tissue is a metabolically active endocrine organ with different body functions (*e.g.* energy and feeding regulation, glucose and lipid metabolism, neuroendocrine function) which impact importantly on cardiovascular system [94]. The adipose tissue achieves these effects through the release of important chemical mediators, named adipocytokines. These include adiponectin, apelin, chemerin, leptin, nesfatin, omentin, resistin, visfatin, and interleukins, including IL6, as well as plasminogen activator inhibitor 1. Here we will consider only adiponectin and briefly apelin, for the other adipocytokines the reader is kindly redirect to specific reviews [43,95].

Adiponectin is one of the most abundant adipokines secreted not only by adipocytes, but also by cardiomyocytes [96,97]. Adiponectin presents two type of receptors expressed in cardiac cells. These receptors structurally and functionally distinct from classical GPCR, have seven transmembrane domains and are named: AdipoR 1 and AdipoR 2. In liver, skeletal muscle and endothelial cells, the activation of receptors induces activation of AMP kinase (AMPK), peroxisome-proliferator activated receptor alpha (PPARα) and p38 mitogen-activated protein kinase (MAPK) [98]. Simultaneous disruption of both AdipoR1 and AdipoR2 causes marked glucose intolerance. The activation of these receptors induces a complex signaling pathway, where probably a central role is played by the activation of AMPK/endothelial NOS (eNOS), while the multiple domain protein APPL1 (adaptor protein with phosphotyrosine binding, pleckstrin homology domains and leucine zipper motif) may be a key mediator [99]. Obesity and type 2 diabetes are correlated with the reduction of adiponectin, while high levels of adiponectin are associated with reduction of CVD [100].

The beneficial cardiovascular effects, including reduction of I/R injury, induced by adiponectin are mediated by paracrine/autocrine activation of specific receptors [101] (Fig. 2). The lack of adiponectin, during I/R injury, increases myocardial infarct size and myocardial apoptosis with reduction of contractile performance [102]. The protective effects are correlated to its ability to activate AMPK, and to reduce inflammatory and oxidative/nitrative stress, thus limiting apoptosis and promoting cell survival. In fact in the null mice for adiponectin it has been observed an augmented production of NO and superoxide anion and consequently of peroxynitrite respect to wild-type mice [98,101,102]. The antiapoptotic effect is associated to the formation of the metabolite S1P and it is dependent by the two adiponectin receptors [103]. In the endothelial cells the AdipoR 1 and 2 activation induces NO-dependent vasodilatation and this effect is mediated by *via* AMPK-mediated phosphorylation of eNOS in two sites (Ser1177 and Ser633) [104].

Adiponectin induces the inhibition of ROS production with an AMPc/PKA/AMPK, mechanism and limits oxidized LDL and palmitate induced apoptosis in *in vitro* model. Yet in the marrow-derived endothelial progenitor cells (EPCs), adiponectin stimulates survival, proliferation, migration and differentiation *via* PI3K/Cdc42/Rac1 signaling cascade [105,106]. Important protective effects of adiponectin are mediated by a PPARγ dependent mechanism leading to increased expression and secretion of adiponectin. This mechanism protects the heart from hypertrophy and from hypertrophic signals induced by angiotensin II *via* Akt/GSK3β/β-catenin and Akt/mTOR pathways [97,107].

The adipocytokine, ***apelin***, activates PI3K/Akt and ERK1/2 in various tissues and, therefore, it has been demonstrated that when administered at reperfusion at pharmacological concentrations induces cardioprotection *via* RISK pathway and the prevention of mPTP opening [108,109].

Bradykinin

Bradykinin (BK) is an important cardioprotective factor. BK and Lys-bradykinin (Lys-BK; kallidin) are the main kinin peptides produced by the action of serine proteases kallikrein kinin system. These two peptides are potent and efficient vasodilatatory agents, particularly active on peripheral and coronary arteries. Their vasodilator effect depends on three mechanisms: *i)* the release of NO, prostacyclin and endothelium derived-hyperpolarizing factor, and the inhibition of endothelin release from the endothelium, *ii)* inhibition of superoxide anion production and *iii)* inhibition of catecholamine release from the sympathetic nerve terminals in the arteries [110]. The BK effects are mediated by the activation of specific BK receptors (B1 and B2 receptors).

Several experimental models highlighted the important role of BK in cardioprotection, both in ischemic PreC and in PostC models [111-114]. In particular, Wall *et al.* [111] were among the first to show that the protective effect of BK is due to B2-receptors activation. In fact the antagonist of these receptors, HOE 140, blocked the effect of ischemic PreC, thus suggesting that BK was among the factors released by ischemic PreC stimuli. These results were confirmed in other animal models of conditioning, where these receptors have been shown to play a crucial role for cardioprotection [112-114]. The data regarding BK are more controversial in humans. Importantly, in a randomized study, patients undergoing isolated coronary artery bypass grafting, BK infusion prior to coronary artery bypass lowered creatinine kinase-MB release, though the troponin I levels in these patients were comparable to those measured in control group [115]. However, systemic administration of a BK B2 receptor antagonist did not affect the endothelial vasomotor dysfunction induced by I/R injury or the protection triggered by remote ischemic PreC [116].

Nevertheless, the importance of BK in clinical arena arises from the well know effect of angiotensin-converting enzyme inhibitors (ACE-I), which induce accumulation of BK. In fact some studies aiming to protect the heart obtained positive results using ACE-I alone [117,118] and other studies suggested that ACE-I may enhance the protective effects of a sub threshold conditioning stimulus [119,120]. Several large clinical trials with ACE-I have shown good out-comes with the

administration of these drugs after AMI [121]. For instance, in several small clinical trials the ACE-I *enalaprilat* administered directly into the coronary artery during reperfusion improved ST-segment elevation, ventricular repolarization, arrhythmias, and inflammation, even in patients unresponsive to ischemic PreC [122]. Taken together, these data, in particular in light of its relationship with ACE system, make studies on BK particularly intriguing, deserving further investigations.

Glucagon-like peptides (GLP-1 and GLP-2)

Glucagon-like peptides 1 and 2 (GLP-1 and GLP-2) are two incretins, which comprise several gastrointestinal hormones with multiple actions, including cardioprotection. They stimulate a decrease in blood glucose levels, whose glucose-dependent insulin tropic actions have been exploited as a novel therapy for glycemic control in type 2 diabetes. Together with GIP (glucose-dependent insulinotropic peptide, also known as gastric inhibitory peptide), GLP-1 represent one of the principal incretin hormones in humans.

Glucagon-like peptide-1. GLP-1 is released from the L-cells of the small intestine and is implicated in the regulation of satiety and appetite. GLP-1 acts through GLP-1 receptor, a member of the GPCR superfamily composed of 463 amino-acids. This receptor is widely distributed in the organism. It can be find in brain, kidney, lung, pancreas, gut and stomach. The intere form of the active peptide, GLP-1 (7–36), presents metabolic effects, in particular induces glucose-dependent insulin release and avoids glucagon to regulate glucose homeostasis. The enzyme dipeptidyl-peptidase-4 swiftly catabolizes GLP-1 (7–36) to GLP-1 (9–36), which may activate GLP-1 receptor, or possibly a second unidentified receptor [123]. GLP-1(7-36) amide and the GLP-1 receptor agonist, exendin-4, induce an increase in blood pressure and heart rate in either conscious restrained or anesthetized rats, but the mechanisms are unclear [123,124]. In several models GLP-1 significantly reduced myocardial I/R injury with reduction of contracture; these protective effects were abolished by administering a GLP-1 antagonist, such as exendin (9-39), or inhibitors of PI3K [125,126]. During low flow ischemia, GLP-1 and insulin-mediated glucose uptake do not involve Akt-1 activation, but an increase in p38 MAPK activity, which is responsible for cardioprotective effect in the post-ischemic myocardium [125,127]. In fact, studies with specific inhibitors confirmed that the intracellular pathways involved in GLP-1-induced protection include p38/NO and p70s6K [126,128]. Administration of the NOS inhibitor, L-NG-Nitroarginine Methyl Ester, attenuated this protective effect in mouse model, pointing to GLP-1-mediated cardioprotection through modulation of NO. In fact, GLP-1 exerts a dose-dependent vasodilator action, which is mediated by endothelial factors, including eNOS-dependent NO release [129,130]. However, also endothelium-independent activation of KATP channels or AMP have been proposed as important mechanisms of GLP-1 vasodilator effects [131,132]. Another pathway described for GLP-1-induced cardioprotection is the inactivation of the so-called *endoplasmic reticulum stress signaling pathway*, which comprises a decrease of both TNF receptor associated factor 2 (TRAF2) and activating transcription factor 4, as well as

the down-regulation of caspase-3 and Grp78 [133]. More recently, the important role of GLP-1 in CVD was confirmed in both preclinical and clinical studies [123].

Glucagon-like peptide-2. GLP-2 is a 33-amino acid peptide, it is an appetite-inhibiting hormone that affects multiple aspects of intestinal physiology, including growth, barrier function, digestion, absorption, motility, and blood flow [134]. GLP-2 induced-pathway involves a unique signaling mechanism and multiple indirect mediators. The GLP-2 receptor is highly homologous to GLP-1 receptor. It is coupled to Gs and Gi/o proteins, and mediates both proliferative and antiapoptotic cellular responses. Recently, Angelone *et al.* [135] have shown that the heart expresses receptors for GLP-2 in basal conditions. Exogenous GLP-2 induced dose-dependent coronary constriction, as well as inotropic and lusitropic effects. In particular, the cardiovascular effects by GLP-2 were due to Gi/o proteins and involved phospholamban and ERK1/2. The infusion of GLP-2 at concentrations which are in the physiological range induced dose dependent biphasic effects: while at a very low dose (10^{-12} mol/L) GLP-2 stimulated contractility, a higher concentration (10^{-10}mol/L) reduced contractility and the rate of relaxation. Contrarily to intestinal cell, the cardiovascular effects induced by GLP-2 were independent from the eNOS/NO system and Akt phosphorylation. The inotropic and lusitropic negative effects induced by GLP-2 were dependent by reduction of phospholamban-(Ser16) phosphorylation [135]. Recently, we reported that GLP-2 infused in the early reperfusion induces a cardioprotective/PostC like effect. GLP-2 PostC limited infarct size and improved post-ischemic recovery of cardiac function. In particular GLP-2 induced an increased phosphorylation of RISK kinases (Akt, ERK1/2 and GSK3β) and favored the opening of mKATP channels. The co-infusion with a specific inhibitor for PI3k/Akt, wortmannin, abolished the cardioprotective effects and the phosphorylation of all studied kinases [136]. Of note, pre- and post-ischemic treatment with GLP-2 attenuated also intestinal I/R injury, reduced bacterial translocation, inhibited the release of ROS and endothelin-1, and abolished the production of proinflammatory cytokines [137].

Sphingosine-1-phosphate (S1P)

Sphingolipids and their metabolizing enzymes are beginning to be recognized as critical mediators in biological processes, specifically in autoimmunity, inflammation and cardioprotection. In particular the membrane sphingolipid *sphingosine* is phosphorylated to S1P by two isoforms of the enzyme *sphingosine kinase* (SK), SK1 and SK2. These enzymes can be activated by numerous growth factors and cytokines, including IL-1β and TNF-α, leading to the generation of S1P. It seems that SK1 is the isoform associated with cell survival. Many actions are mediated through S1P-GPCR subtypes, Gαi, namely the $S1P_1$ receptor, which is highly expressed in cardiomyocytes. It has been reported that S1P is released in both PreC and PostC and its binding to $S1P_1$ receptor leads to activation of ERK1/2. $S1P_2$ and $S1P_3$ receptors are also present on cardiac cells coupled with both Gαq and Gαi [138.]. It has been also reported that $S1P_3$ receptor binding leads to the activation of PI3K and Akt. Therefore S1P protection depends on ERK1/2 and in part

on PI3K/Akt of the RISK pathway in the heart [59,139]. Nevertheless, S1P protection is also due to the SAFE pathway. In fact, the protective effects of TNF-α (an element of SAFE pathway) are attenuated by inhibitors of the sphingolipid pathway [140]. Actually, the downstream target of TNF-α, TRAF2, induces intracellular formation of S1P *via* upregulation of SK1 [58]. Moreover, S1P activates STAT3 through the $S1P_2$ receptor, thus inducing ERK1/2 and then STAT3 activation [59,139]. Recently it has been reported that the levels of phosphorylated STAT3 of SAFE pathway are significantly increased in both the nuclear and mitochondrial fractions in the S1P pre-treated hearts [141]. Therefore, S1P is a trigger for RISK and SAFE pathways and it may be a novel therapeutic target to modulate mitochondrial and nuclear function in CVD in order to protect the heart against I/R.

Opioids

In addition to pain modulation, opioids are involved in several physiological and pathophysiological regulatory processes, including membrane transports, immune function, feeding, cardiovascular and respiratory control; for reviews, see [142,143]. Three main opioid receptor families, μ-opioid (MOR), δ-opioid (DOR), and κ-opioid receptor (KOR) have been described, each of which sub-classified into several subtypes. Activation of opioid receptors, in particular DOR, has been demonstrated to preserve cellular vitality following a hypoxic insult, such as I/R, in many systems including the central nervous system [144], the intestine [145], the skeletal muscle [146] and the myocardium [147]. For the purpose of this review, we will focus mainly on the protective effects of opioids against myocardial I/R. Various opioid peptides, including dynorphin-like peptides, enkephalins and β-endorphin are present within the heart. Cardiac cells themselves are able to synthesize, to store and to release opioid peptides. In particular, elevated levels of these mediators have been found within the heart in stress conditions, such as ischemia.

Gross group was among the first to show cardioprotective effects by opioids [148,149]. Both DOR1 and DOR2 subtypes have been shown to be involved in PreC of the heart. For example, Lasukova *et al.* [150] showed that a DOR1 agonist exerted antiarrhythmic and cardioprotective effects after I/R. On the other hand, Maslov *et al.* [151] reported that DOR2 blockers abolished the protective effects of deltorphin II, while a DOR1 antagonist had no effect. The effects of deltorphin II were abrogated by both PKC and NOS, as well as by mKATP channels blockers. Notably, the inhibition of tyrosine kinase, hexamethonium (a ganglion blocker) and catecholamine depletion reversed the antiarrhythmic effect of deltorphin II, but did not alter its positive effect on infarct size. Comparable protective effects have been shown also after KOR activation, being able to mediate both the anti-arrhythmic and infarct sparing effects of ischemic PreC, and to attenuate myocardial apoptosis and necrosis in I/R heart [152].

Other studies have associated cardiac opioid receptors to ischemic PostC protection[84,153]. Kin *et al.* [84] using the *in situ* rat heart demonstrated that PostC-induced protection was abrogated in the presence of naloxone, a nonspecific opioid receptor antagonist, suggesting that endogenous opioid binding to its GPCR may also be responsible of the

infarct-sparing effect of PostC. Moreover, Wang *et al.* [153] have shown that KOR are involved in PostC-induced cardioprotection, using the same experimental model used by Kin *et al.* [84].

In conclusion, these data suggest that, together to neuroprotection, DOR-mediated cardioprotection may be a potential useful phenomenon in terms of prevention and treatment of dangerous conditions for life, such as stroke and AMI. Although it is well known that opioid receptor activation during the brief PreC ischemia acts as a trigger for subsequent cardioprotection [149] and that the pharmacological activation of the opioid receptor at the beginning of coronary reperfusion is cardioprotective [154], it remains to be demonstrated whether binding of the endogenous opioid to receptors at time of cardiac reperfusion contributes to conditioning protection.

Platelet-activating factor (PAF)

Several findings support the hypothesis that PAF may play a dual role in I/R injury of the heart; for reviews, see [155-159]. Although the role of PAF in the pathogenesis of myocardial I/R damage is clear, recently it has been demonstrated that very low concentrations of PAF given before an index ischemia exert cardioprotective effects, comparable to those afforded by PreC. PAF belongs to a family of biologically active, structurally related alkyl phosphoglycerides which play an important role in different pathophysiological conditions affecting the cardiovascular system, including cardiac I/R injury. The effects of PAF are due to specific receptors (PAFR), which belong to *GPCRs* superfamily. Since cardiac cells produce PAF and possess PAFR, it is likely that PAF is an autocrine/paracrine mediator. During cardiac I/R, PAF is released in concentrations (1-10 10^{-9} mol/L) high enough to negatively modulate coronary, contractile and electrical activities. In vivo, PAF may act both directly and through the activation of platelets and pro-inflammatory polymorphonuclear neutrophils, which exacerbate post-ischemic cardiac damage. Actually the deleterious effects exerted by high concentrations of PAF are well established. However several experimental data demonstrated that very low concentrations (10^{-12} mol/L) of exogenous PAF given before I/R induce cardioprotective effects akin to those afforded by ischemic PreC, and that endogenous PAF production contributes in the triggering of ischemic PreC itself [160]. The PreC-like action induced by low concentrations of PAF is due to the activation/phosphorylation of PKC, Akt/PKB and NOS, enzymes included in the RISK pathway. These activation/phosphorylations and the activation of mKATP channels may allow the prompt interventions of signaling pathways leading to the prevention of mPTP at reperfusion [161,162]. In addition, it has been shown that low concentrations of PAF increase the basal intracellular Ca^{2+} transients in Ca^{2+}-overloaded cardiomyocytes, thus attenuating their time-dependent loss of shortening [52]. These protective effects of PAF depend on NO production and S-nitrosylation of myocardial proteins, rather than the activation of GC and production of cGMP. NO-induced S-nitrosylation of Ca^{2+} handling proteins, such as L-type Ca^{2+} channels, may be responsible for the reduced Ca^{2+} overload. In conclusion, endogenous synthesis of low levels of PAF during a brief I/R episode may play a pivotal role in the triggering of PreC. It is known that exercise can mimic the

protective effect of PreC. The fact that low quantities of PAF are released in certain conditions, such as during atrial pacing, exercise, or in non-infarcting ischemia, and the fact that these low quanties of PAF may participate in PreC, suggest the potential importance of a moderate release of PAF, as an attempt by the heart to protect itself against I/R injury. Yet, we should consider that therapies for inflammatory diseases may completely block PAFR, thus inhibiting PAF protective action and this may compromise myocardial postischemic function, since a significant, endogenous cardioprotective pathway is disrupted.

Growth hormone-releasing hormone (GHRH)

Growth hormone-releasing hormone not only stimulates growth hormone synthesis and release from the pituitary, but also exerts direct effects in extrapituitary tissues, including the cardiovascular system; for reviews, see [163-165]. Indeed, GHRH was shown to exert cardioprotective effects, being able to limit apoptosis induced by serum starvation or isoproterenol in adult rat ventricular myocytes and in the H9c2 cardiac cell line [166]. The protective effects of GHRH are due to its interaction with specific GHRH-R and require activation of ERK1/2 and PI3K/Akt, as well signaling mediated by adenylyl cyclase/cAMP/PKA. In isolated rat hearts subjected to I/R injury, PreC with GHRH strongly reduced the development of infarct size and improved left ventricle (LV) diastolic pressure recovery (*i.e.* reduced cardiac contracture) following reperfusion. These positive effects were mediated by GHRH-R *via* a PI3K/Akt signaling, as suggested by their inhibition in the presence of specific antagonists, namely JV-1-36 and LY294002 [166]. Further studies from our laboratory were performed to determine whether GHRH is able to reduce myocardial reperfusion injury also when given at the onset of reperfusion, and to investigate the molecular mechanisms involved in GHRH protective effects [167]. Treatment of isolated I/R rat hearts with GHRH at the onset of reperfusion reduced infarct size at the end of reperfusion and reverted contractile dysfunction. The use of specific pharmacological blockers and Western blot analysis showed that the protective effect of GHRH is due to phosphorylation of either the RISK (PI3K/Akt, ERK1/2 and GSK3β), or STAT3, as part of the SAFE pathway.

Furthermore, GHRH increased the phosphorylation of eNOS and AMPK, and preserved post-ischemic NAD^+ levels [167]. Taken together, these results suggest that the protective action of GHRH from I/R injury is due to a receptor-mediated mechanisms, leading to activation of both RISK and SAFE pathways, which converge on mitochondria and possibly on AMPK.

The protective effect of GHRH observed *in vitro* has been confirmed *in vivo* by Kanashiro-Takeuchi *et al.* [168], who demonstrated that JI-38, a potent GHRH agonist (GHRH-A), triggers cardioprotection after AMI. Animals receiving GHRH-A displayed reduced infarct size and improved cardiac structure and function. In addition, GHRH-A increased Bcl-2 antiapoptotic protein in cardiomyocytes and reduced cardiac fibrosis . More recently, Kanashiro-Takeuchi *et al.* [169] showed that GHRH-A also enhances functional recovery and reverses ventricular remodeling in the setting of

chronic myocardial infarction. In both studies, circulating levels of GH/insulin-like growth factor (IGF)-I were not increased by GHRH-A, suggesting that GHRH-A exerts direct GH/IGF-I independent effects. In addition, the use of a GHRH antagonist blocked GHRH-A-induced cardioprotection, indicating receptor-mediated mechanisms [169].

Tyrosine kinase receptor activators

Erythropoietin

Erythropoietin (Epo) is a 165 amino acids glycoprotein hormone known for its effect on stimulation of proliferation and survival of red blood cells progenitors [170]. Epo is mainly produced in the kidney and liver; however its mRNA, as well as Epo receptor (EpoR) expression, have been shown in many tissues, including the heart, suggesting Epo pleiotropic actions [171].

Epo exerts cardioprotection through antiapoptotic and antihypertrophic effects, as well as mobilization of progenitor cells from the bone marrow [172,173]. Indeed human recombinant (hr)Epo was shown to limit infarct size and LV remodeling in animal models of AMI through antiapoptotic, anti-inflammatory and angiogenic effects [174,175]. The antiapoptotic effects were associated with activation of PI3K/Akt and ERK1/2 pathways. Interestingly, even endogenous Epo-EpoR system plays a protective role in cardiac I/R, at least in part by preventing apoptosis and reducing ROS production [176,177]. The mediator of inflammatory and cytokine response NF-κB was also involved in Epo signaling, thus participating to Epo-mediated cardioprotection. Moreover, EpoR activates STAT3 and p38 MAPK, and increases vascular endothelial growth factor production, therefore increasing angiogenesis [177]. Collectively, these studies, among others, indicate that Epo-induced cardioprotection is likely due to its antiapoptotic, antinflammatory, and proangiogenic effects.

Additional actions of Epo may also contribute to cardioprotection. In fact, Epo was found to enhance cardiomyocyte contractility [178] and to stimulate neuronal and epithelial progenitor cell proliferation [179], suggesting that the hormone may promote the mobilization of non-differentiated stem cells into ischemic area of the myocardium [180]. Epo was also found to act with retinoic acid to support myocardial proliferation in the intact embryonic heart [181] and to promote the proliferation of cultured neonatal myocytes [182].

Based on the encouraging results obtained in animal models, it was suggested that EpoR activation would be a promising target for the treatment of AMI. However, several other studies conducted in both animals and humans provided conflicting results. This was likely because either the dose of hrEpo or the timing of its administration were inappropriate. It was suggested that the dosage of hrEpo, the therapeutic window, the duration of treatment and the experimental model used to replicate AMI in both animal and human studies all had a role in producing conflicting results in heart failure and AMI [171].

Importantly, new clinical trials are being performed, also using new EpoR agonists devoid of erythropoietic activity, which still exert cardioprotective actions and should lead to more promising effects, in the attempt to translate the cardioprotection found in experimental models to human patients [183].

Thrombopoietin

Thrombopoietin (TPO) is a humoral growth factor, which was originally identified for its capacity to induce megakaryocytes differentiation and proliferation. It is produced by the liver and the kidneys, and subsequently cleared from circulation after binding with its receptor c-Mpl, mainly expressed on megakaryocytes and platelets [184,185]. Several data point out the role of TPO in different pathophysiological processes involving the heart. For instance, high levels of circulating TPO have been reported in several clinical conditions, such as sepsis and acute coronary syndrome [186]. TPO is able to negatively modulate myocardial contractility, by acting on its receptor c-Mpl on cardiomyocytes and stimulating NO synthesis. Moreover, TPO toghether with IL-1β and TNF-α is responsible of the cardiodepressant effects exerted *in vitro* by serum of septic shock patients [187]. Interestingly, TPO may also act as a physiological regulator of coronary flow, by acting on receptors present on endothelium and inducing PI3K/Akt dependent eNOS phosphorylation and NO synthesis [188]. In addition, TPO may exert a protective effect on the heart under I/R or oxidative stress.

Indeed, it has been shown that TPO pre-treatment reduced apoptosis and myocardial necrosis, as well as ventricular dysfunction following I/R both *in vivo* and *in vitro* in the rat isolated heart. These effects were mediated by Janus kinase (JAK)-2, p42/44 MAPKs, and KATP channels [189].

The anti-ischemic protective effects of TPO have been recently confirmed by Chan *et al.* [190] on rats subjected to left coronary artery ligation and treated with TPO immediately after induction of AMI and during the 2 following weeks. Four or 8 weeks after the induction of AMI, TPO treatment significantly reduced infarct size and improved left ventricular function and hemodynamic parameters, myocardial morphology and neovascularization. TPO treatment also reversed the alterations in the expression of genes, such as those involved in cytoskeleton organization, vascular and matrix remodeling, muscle development, cell cycling and ion transport, observed in the infarct border zone. Moreover, TPO regulated phosphorylation of STAT3 and ERK, bone morphogenetic protein 1 level, and mobilized EPC colonies in the bone marrow of AMI animals [190].

TPO exerted an antiapoptotic effect in two different *in vitro* models: the primary neonatal rat cardiomyocytes and the fetal rat cardiomyocyte cell line H9c2. It also protected against cardiac toxicity induced by doxorubicin. In particular, in an *in vivo* model of doxorubicin-induced acute cardiotoxicity, TPO preserved heart rate, fractional shortening and cardiac output [191]. These previous results have been recently confirmed by studying the effects of TPO in both acute- and chronic-doxorubicin treatment rat models [192]. In both cases, TPO reduced the deleterious effects of doxorubicin

on cardiac performance and morphologic parameters. TPO also counteracted the altered expressions of some genes, including modulators of ion transport, antiapoptosis, Akt/ERK pathways, signal transduction, cell division, contractile function and protein/matrix remodeling. Also in this case, TPO increased the formation of EPC colonies in the bone marrow. These data suggest that TPO-induced cardioprotection from doxorubicin injury is due to several mechanisms, in particular Akt- and ERK-dependent restoration of regulatory gene activities that are crucial for normal cardiac function.

Insulin

The studies on insulin mechanisms of action have been classically focused on the metabolic effects of the hormone and its role in the pathophysiology of insulin resistance and diabetes, with major attention on insulin-sensitive targets such as skeletal muscle, adipose tissue, liver, and brain.

The *insulin receptor* is expressed in a variety of organs, including the heart, indicating pleiotropic actions of insulin. Indeed, insulin signaling has been linked with both cardiovascular physiology and pathological conditions associated with insulin resistance and diabetes, including diabetic cardiomyopathy, coronary artery disease and myocardial ischemia, which ultimately lead to heart failure [193]. A prominent feature of the diabetic myocardium is cardiac hypertrophy, with changes in LV structure and function and by compromised systolic and diastolic function, in the absence of active ischemia. Diabetes-associated hyperglycemia, increased circulating fatty acids and inflammatory cytokines and iperinsulinemia all impair cardiac contractility and cause cardiomyocyte dysfunction and death.

In the heart, insulin induces glucose uptake into cells, by binding to its receptor and mainly through the contractile mediated translocation of the glucose transporters GLUT4 and GLUT1 [194]. Moreover, insulin-induced activation of PI3K/Akt regulates other cellular processes, such as hypertrophy, protein translation, NO generation, apoptosis and autophagy, by activating other intracellular signaling intermediates such as mTOR, S6K, forkhead transcription factors (FOXO) and GSK3β. Insulin resistance-induced changes of these signaling pathways may contribute to the developing of cardiac hypertrophy, LV dysfunction and chronic heart failure (CHF) [195]. Insulin also promotes the translocation of the fatty acid transporter CD36 and targets fatty acids to the triglyceride pool. Overall, insulin increases glucose metabolism and reduces fatty acid oxidation. It also regulates mitochondrial oxidative capacity and the adaptation of mitochondria to physiological cardiac hypertrophy [196,197], through mechanisms involving Akt-mTOR-NFκB signaling [198]. Insulin signaling is required for preserving myocardial function and structure in response to pressure overload hypertrophy and ischemia [199-201]. On the other hand, excessive insulin signaling has also been implicated in accelerating LV remodeling in CHF and was found to prevent ischemic PreC [202,203].

Constitutive activation of Akt, a target of different cardioprotective hormones, including insulin [204], was found to preserve cardiac function and to prevent injury after transient cardiac ischemia *in vivo* [205,206]. Moreover, a recent

study showed that in a porcine model of acute coronary syndrome, intracoronary insulin, administered at the onset of reperfusion, reduced regional myocardial dysfunction and reduced myocardial apoptosis [207].

In conclusion, the main role of insulin in the heart under physiological conditions is the regulation of substrate utilization. Indeed, insulin promotes cardiomyocyte contraction, increases protein synthesis, stimulates vascular endothelial growth factor and angiogenesis, suppresses apoptosis, promotes cell survival and ameliorates both myocardial microcirculation and coronary artery resistance, leading to increased blood perfusion of myocardium. Conditions such as type 2 diabetes, myocardial ischemia, and cardiac hypertrophy have been shown to alter insulin signaling and insulin actions, further exacerbating the disease states.

Growth factors

Many growth factors protect the heart from the detrimental effects of I/R injury, through the activation of a variety of cell-surface receptors and the recruitment of intracellular signal transduction pathways, including RISK pathway. Here we review a couple of these factors with respect to their ability to confer myocardial protection, whose mechanisms have also been studied in our laboratories. For the other growth factors the reader is kindly redirect to the excellent review of Housenloy and Yellon [208].

Neuregulin

In the adult heart neuregulin-1 (NRG1) is synthesized by both endocardial and microvascular endothelial cells [209], but its release was recently shown also in coronary artery endothelial cells in culture [210]. Cardiomyocytes express ErbB2 and ErbB4 receptors, and the endothelial derived NRG1 directly binds to ErbB4, inducing a conformational change which leads to the formation of ErbB4 homodimers or ErbB4/ErbB2 heterodimers [211].

The NRG1/ErbB axis is required for cardiac development and in the postnatal adult heart it exerts several pro-survival effects against oxidative stress and cytotoxic agents [212]. The prominence of the protective role of this paracrine pathway in the myocardium is clearly highlighted by the well-known cardiotoxicity of Trastuzumab, an ErbB2 binding antibody used in cancer therapy [213].

Several studies on both neonatal and adult cardiomyocytes reveal that the beneficial effects of NRG1 mainly involve regulation of protein synthesis and sarcomere stability, regulation of cellular metabolism and growth, maintenance and repair of electro-mechanical coupling [214,215]. Moreover, it has also been shown that NRG1 improves diastolic calcium handling by the activation of PI3K, eNOS and PKG [212,216].

Recently, NRG1 has also been proposed as an important cardioprotective agent against myocardial I/R damage. First evidences for this role came from a study by Kuramochi *et al.* [217], showing that ROS activate NRG-1beta/erbB4

paracrine signaling, both *in vitro* (cocultured adult rat cardiomyocytes and cardiac microvascular endothelial cells) and *ex vivo* (isolated mouse hearts subjected to oxidative stress by I/R injury).

Moreover, NRG1 protection against I/R was also observed in adult murine cardiomyocytes cocultured with human umbilical vein, murine lung microvascular, or human coronary artery EC and confirmed *in vivo* in mice, where endothelium-selective deletion of NRG1 leads to a significantly decreased tolerance to ischemic insult [210].

NRG1 also revealed significant PreC properties in an *in vivo* rat model of I/R [218]. This study demonstrated that NRG1 was upregulated after myocardial I/R injury and that NRG1 PreC reduced infarct size and apoptosis, by a PI3K/Akt-dependent mechanism.

The same PI3K/Akt pathway was also involved in NRG1 dependent PostC, as shown in an *ex vivo* mouse model of I/R injury when NRG1 was infused starting 5 min prior to reperfusion [219]. Interestingly, in these experiments NRG1-dependent cardioprotection required eNOS inhibition, suggesting that, during ischemia, superoxide production by uncoupled eNOS annihilates NRG1 PostC. As dysfunctional eNOS occurs in several pathological conditions like diabetes, hypertension and hypercholesterolemia, this study could explain the inefficacy of PostC in models of cardiovascular comorbidities.

The promising animal studies have led to ongoing clinical trials. A phase II, double-blinded, placebo-controlled study has been performed to assess the safety and efficacy of recombinant human NRG-1 in CHF patients. The results showed that recombinant human NRG-1 improved the cardiac function of CHF patients and showed its antiremodeling capability by decreasing end-systolic more than end-diastolic LV volume, thus increasing the LV ejection fraction (LVEF) compared with pre-treatment [220]. These encouraging researches led to further investigate the potential therapeutic application of NRG1 in CHF and to discover useful strategies to increase this endogenous paracrine signal to limit I/R injury.

Ghrelin-associated peptides

Small peptides GH secretagogues (GHSs) promoting GH-release from the pituitary were initially discovered in 1976 and their receptor (GHS-R) cloned in 1996 [221]. Its endogenous ligand ghrelin is a 28 amino acid peptide initially isolated from the stomach, whose acylation is essential for binding to GHS-R type 1a and its endocrine functions, including stimulation of GH secretion and food intake [221]. Although lacking GHS-R binding, *unacylated ghrelin* is another ghrelin form, which shares several effects with *acylated ghrelin*. Ghrelin exerts different activities on cardiovascular system, such as inhibition of cardiac and endothelial cells apoptosis [222], modulation of cardiac contractility through an endothelial cells-NO dependent mechanism [223], and improvement of left ventricular function in I/R heart [224]. Ghrelin attenuated cardiac dysfunction and development of cachexia in rats, as well as in patients with CHF, in which also decreased systemic vascular resistance [225]. In addition, ghrelin was recently found to

directly modulate cardiac energy substrate metabolism by enhancing free fatty acid oxidation and reducing glucose oxidation in heart failure dogs, thus partially correcting metabolic alterations in heart failure [226].

Obestatin, a recently identified novel ghrelin gene peptide, may also play a significant role in regulating cardiac function in humans, in both physiological and pathological conditions. Actually, higher saliva obestatin levels were found in obese patients with ischemic cardiac disease compared with healthy individuals [227] while decreased levels were present in serum of patients with both obesity and type 2 diabetes mellitus [228]. Moreover, an altered ghrelin to obestatin ratio has been shown in spontaneously hypertensive rats and in CHF patients with cachexia [229,230]. Recently, the serum levels of ghrelin were found to be positively correlated with *Angiotensin II* in CHF individuals. Interestingly, ghrelin by down-regulating angiotensin 1 receptor inhibited Angiotensin II-induced apoptosis of cardiomyocytes *in vitro*, thus playing a role in preventing heart failure [231].

The protective effects of obestatin against I/R injury were recently investigated [232]. Pretreatment of isolated rat hearts with obestatin reduced contractile dysfunction and infarct size induced by I/R. Moreover, obestatin exerted an antiapoptotic effect on isolated ventricular myocytes or H9c2 cells subjected to I/R. These cardioprotective effects were due to activation of PI3K, PKC, or ERK1/2 pathways. Specific high-affinity obestatin binding sites were mainly localized on membranes of ventricular cardiomyocytes. Although it has been proposed that obestatin opposes several effects of ghrelin, the protective action of obestatin observed was fully comparable to that induced by ghrelin on the same experimental models [224]. In the myocardium of STZ-treated diabetic rats obestatin exerted a significant beneficial on contractility and ß-adrenergic response *via* the activation of pro-survival signaling pathways [233].

In conclusion, several evidences indicate that GH secretagogues are emerging as cardioprotective agents. In addition to ghrelin, obestatin, the new described peptide of the ghrelin gene family, demonstrated its ability to play a relevant role in cardiac function and protection. Although future studies are necessary to evidence the mechanisms underpinning the effects of these peptides, the reported findings suggest their potential therapeutic use in cardiac dysfunctions induced by I/R.

Guanylyl cyclase(GC)-linked receptor activators

Natriuretic peptides

Atrial (ANP), brain (BNP) and C-type natriuretic peptide (CNP) are polypeptide hormones secreted from the heart as a result of direct wall stress, caused either by stretch or pressure affecting cardiomyocytes, to protect the human body from a volume overload [234]. Also hypoxia was a direct and sufficient stimulus for ANP release from an isolated rodent heart [235] and hypoxia- sensitive elements were found from the promoter sequence of both the ANP and the BNP genes [236,237]. ANP is usually synthesized in the atria, BNP is primarily synthesized in the ventricles and CNP is predominantly produced by the endothelium. Upon their release, natriuretic peptides (NPs) act at multiple sites to

exert diuretic, natriuretic and vasorelaxant effects. Moreover, ANP, BNP and CNP also act as paracrine factors, inducing antihypertrophic and antifibrotic effects in the heart [238].

NPs exert their hormonal and paracrine effects by binding to three distinct cell surface receptors, the guanylyl cyclase-linked -A and -B receptors (GC-A, GC-B) and the NP-C receptor. The cGMP produced downstream of GC-A/B stimulation can modulate several targets (including PKG and phosphodiesterases 2 and 3). NP-C receptor, which binds all NPs, is not directly linked to a GC enzyme, but is rather coupled to the activation of inhibitory G proteins (Gi) [234,238]. Both cGMP and Gi dependent pathways have been associated to cardioprotection: several studies report that cGMP-induced signaling inhibits hypertrophy, decreases fibrosis, and protects against cardiac I/R [239]. Moreover, upregulation of Gαi2 in ischemic myocardium appears to be protective in inducing cell survival pathways preventing myocyte death in response to I/R injury [240].

According to these results, several experimental evidences in different animal models confirm that NPs might protect the heart against infarction when given just prior to reperfusion [241,242]. In particular, it has been shown that administering ANP in rabbit hearts at reperfusion reduced infarction size by activation of PKG, opening of mKATP and stimulation of downstream kinases [75]. Moreover, infusion of CNP prior to or following an ischemic event reduces the size of ventricular infarction by up to 50%, as a result of inhibition of ICa,L, reduction in heart rate and augmented coronary vasodilation [243].

Conversely, other experiments [244] showed that at 30 days postinfarction the survival of ANP-/- mice was markedly better respect to $ANP^{+/+}$ mice, that exhibited increased P-selectin, neutrophil infiltration, infarct size and mortality. As in these experiments mice were subjected to permanent ischemia, the authors suggested that ANP may exert different effects after brief versus prolonged ischemia, two conditions able to activate distinct pathways in the heart [245].

NPs paracrine communication also appears to be involved in cardiac regeneration, as the endothelial GC-A receptor for ANP and BNP is critically involved in cardiac angiogenesis accompanying compensated pressure load–induced hypertrophy [246].

Furthermore, several trials were conducted to determine the beneficial role of ANP/BNP infusion on AMI; however, the results were conflicting. In order to provide a more robust evaluation of the benefits of ANP/BNP in AMI patients, a meta-analysis from 20 trials was recently performed [247]. The results indicated that NPs infusion could improve LVEF% as compared with control, although it is still premature to conclude that ANP/BNP could be safe respect to renal function deterioration and hypotension.

Agents Acting on Intracellular Pathways

Gasotransmitters (NO, H_2S and CO)

The gasotransmitters NO, hydrogen sulphide (H_2S) and carbon monoxide (CO), modulate many physiological functions [248-250]. In particular, in the cardiovascular system, CO, NO, and H_2S induce vasorelaxation, stimulation of angiogenesis, and cardioprotection (Fig. 3) [249,251].

Nitric oxide

In the myocardium, NO is generated by both enzymatic and non-enzymatic reactions. Three NOS isoforms have been identified, neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) [252]. NOS activity is regulated by cellular compartmentalization, availability of substrates and cofactors, endogenous inhibitors, transcriptional, posttranscriptional, and posttranslational modulations. The production of NO by NOS-independent enzymatic and non-enzymatic reduction of nitrite/nitrate from dietary and endogenous sources is of primary relevance during ischemia, a condition characterized by acidic pH and limited oxygen-dependent NOS activity. The main biological reactions of NO include oxidation to nitrite/nitrate and its reactions with anion superoxide to produce peroxynitrite ($ONOO^-$), a reactive nitrating/nitrosating agent. NO targets metalloenzymes (in particular soluble GC, hemoglobin, and cytochromes), with thiols S-nitrosylation/S-nitrosation, producing S-nitrosothiols (RSNOs) [250,253-255].

NO exerts its cardioprotective role by means of the cGMP/PKG pathway, as a fundamental molecule in the RISK and SAFE cardioprotective signaling pathways, and *via* S-nitros(yl)ation of proteins such as SERCA and several mitochondrial proteins [38,48-50,250]. During ischemia, there is accumulation of NO generated by both enzymatic and non-enzymatic sources. Then, upon reperfusion, characterized by an abrupt increase of ROS, NO may be converted to peroxynitrite, thus contributing to *reperfusion injury*. By decreasing peroxynitrite formation, administration of NOS inhibitors at high doses before ischemia may decrease I/R injury. On the other hand, PreC by brief cycles of I/R leads to small increases in NO and peroxynitrite in the trigger phase, leading to a decreased generation of NO and peroxynitrite after infarcting I/R, thus producing cardioprotection [50,253,254]. Indeed, the cardioprotective effect of PreC is lost once NOS is inhibited or in NO deficient conditions such as hyperlipidemia, sensory neuropathy, diabetes,: intact basal myocardial NO generation is needed to achieve cardioprotection by PreC [250,254]. On the other hand, recent experimental studies have shown the usefulness of strategies aimed at increasing cardiac NO metabolites storage before I/R, such as exercise [256,257] or remote preconditioning [258].

Endogenous NO and peroxynitrite are also involved in ischemic PostC. In isolated murine hearts, infarct size was reduced by ischemic PostC, and the eNOS inhibitor L-NG-Nitroarginine Methyl Ester blocked such effect [10,259]. Cardiac peroxynitrite was increased in ischemic PostC in rat hearts. Interestingly, the compound 5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrinato iron (FeTPPS, a peroxynitrite decomposition catalyst) inhibited the cardioprotective effect of PostC on infarct size [260]. Similarly, Li *et al*. [230] showed that when administered before PostC, FeTPPS abrogated its beneficial effect. This suggests that at early reperfusion, NO/ROS interactions contribute to PostC

cardioprotection. Conversely, ischemic PostC even at late reperfusion reduced infarct size in rodents and humans with a reduction of post-ischemic myocardial iNOS activity and generation of nitrotyrosine [250]. Such effect was mimicked by iNOS inhibition, while 3-morpholinosydnonimine abrogated the effects of PostC [261]. Thus, increased NO-peroxynitrite signaling plays a pivotal role in triggering cardioprotection by PostC, which then lowers nitro-oxidative stress in the following full reperfusion, hence contributing to cardioprotection. We have suggested that an initial NO-peroxynitrite signaling is followed by an increase in S-nitrosothiols formation in reperfusion phase [38].

Interestingly, infarct size can be reduced independently from PKG by administering MitoSNO during reperfusion [262]. MitoSNO protects murine hearts *in vivo* against I/R injury by S-nitros(yl)ation of mitochondrial complex I. Indeed, reversible S-nitros(yl)ation of complex I can slow the reactivation of mitochondrial function during reperfusion immediately after ischemia, hence diminishing ROS generation, oxidative damage and tissue necrosis, indicating that rapid complex I reactivation contributes to I/R damage [263]. Beside respiratory complexes, ischemic postconditioning can cause S-nitros(yl)ation of other proteins, which can be blocked by L-NAME, causing a loss of cardioprotection. Moreover, seventy-seven unique proteins with S-nitros(yl)ation sites only modified by PostC have been identified [264].

Hydrogen sulphide

H_2S can be produced endogenously and is present in several cells and tissues in physiological conditions. The main source of H_2S in mammals is desulfhydration of cysteine, catalyzed by cystathionine gamma-lyase (CSE), cystathionine beta-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3- MST). Different molecular targets, such as different ion channels and signaling proteins mediate the physiological functions of H_2S. Alterations of H_2S metabolism are involved in a variety of pathophysiological conditions such as hypertension, atherosclerosis, CHF, diabetes, cirrhosis, inflammation, sepsis, neurodegenerative disease, erectile dysfunction, and asthma [250,265,266].

Endogenous H_2S may modulate cardiovascular function as a potential endogenous gasotransmitter. The effects are mediated by protein S-sulfhydration (a reaction of cysteine residues with H_2S) and activity modification of signaling pathways. The myocardium expresses all three described enzymes that produce H_2S, but most of the study has focused on of H_2S generated by CSE (the most abundant trans-sulfuration pathway enzyme [267]). Nevertheless, the compounds DL-propargylglycine (PAG), or -cyano-L-alanine used for inhibiting H_2S synthesis are selective CSE inibitors, allowing other trans-sulfuration pathway enzymes, CBS and 3-MST, to continue H_2S production .

PAG inhibition of CSE was able to blunt H_2S and increase infarct size in isolated rat hearts [268]. In the same model, exogenous L-cysteine was able to blunt I/R injury through a mechanism dependent on H_2S generation [269]. Also, cardiac-specific over expression of cystathionine-lyase modulated the generation of produced H_2S and attenuated the

extent of injury [270]. Also, myocardial I/R injury was exacerbated in mice lacking CSE; conversely, it was blunted by H_2S [271].

Ischemic PreC necessitates of endogenous H_2S. In ventricular myocytes, ischemia lowered H_2S generation, while this was restored by PreC, showing that endogenous H_2S production is crucial in cardioprotection [272]. In rat isolated myocytes, the cardioprotective effects of ischemic PreC on cell viability/morphology was reversed by pharmacological inhibition of CSE inhibition [273]: lowering H_2S generation blunted protection from PreC [272].

Ischemic PostC also requires endogenous H_2S: H_2S-producing enzymes are activated in the early phase of reperfusion by ischemic PostC [274], whose cardioprotective effects are blunted by PAG [274]. Furthermore, cardiac-specific over expression of cystathionine gamma-lyase regulated the generation of H_2S and reduced myocardial damage [270,275].

The fundamental role played by CSE in the modulation of the I/R injury is further underlined in a recent study from the Lefer Lab [276]. CSE knockout mice showed elevated oxidative stress, dysfunctional eNOS, lower NO levels, and exacerbated myocardial and hepatic I/R injury, while acute H_2S therapy restored eNOS function and NO bioavailability, blunting I/R injury. Interestingly, eNOS phosphomutant mice (S1179A) were not protected against I/R by H_2S administration, suggesting that H_2S protective signaling in the setting of I/R injury is largely dependent on eNOS activation and NO production, further corroborating the evidence on H_2S-NO interaction in the cardiovascular system.

Carbon monoxide

Endogenous CO is an important, biologically active molecule. Heme oxygenases (HO-1 and HO-2) generate CO as a result of the degradation of heme, with formation also of ferrous iron (Fe^{2+}), and biliverdin, which is rapidly reduced to bilirubin, a reaction made possible in the presence of O_2 and NADPH. Such a key reaction for iron and bile metabolism leads to the production of bilirubin, which is an important antioxidant. HO-1 and HO-2 are expressed in both atrial and ventricular cardiomyocytes. HO-1 expression is inducible (it is also known as heat shock protein 32), whilst HO-2 expression is constitutive [250], as in many other tissues.

Signaling pathways comprising NO/GC, ROS and MAPKs are regulated by CO. HO is crucial in cellular antioxidant defense and vascular protection, and it may mediate pleiotropic actions of cardiovascular therapeutics [251]. HO-1 is upregulated in the heart after I/R and provides cardioprotection, with CO being responsible for most beneficial antiapoptotic and cytoprotective effects [277,278]. Additionally, CO also exerts anti-inflammatory and anti-hypertensive effects [251].

HO-1 expression and activity were enhanced by hypoxia and gradually declined during reoxygenation with consequent increase of damage. Interestingly, such injury was reduced by incubation with hemin or bilirubin during hypoxia. Also, hemin and bilirubin could attenuate ROS production: hypoxic cardiomyocytes are protected by the HO-1-bilirubin pathway against reoxygenation injury [279]. Additionally, gene delivery of hypoxia-inducible factor-1 alpha (HIF-1

alpha) induced cardioprotection *via* the downstream factor HO-1: HL1 cells were protected by HIF-1 alpha and HO-1 against H_2O_2-induced damage. The cardioprotective effects exerted by gene delivery of HIF-1 alpha depended on HO activity suggesting that downstream to HO-1, carbon monoxide as well as bilirubin may be organ effectors [280].

Moreover, HO-1 deficient mice are more susceptible to I/R injury, since they develop right ventricular infarction after prolonged hypoxia, while the myocardium is protected from I/R injury by HO-1 over expression [281,282]. In Dahl salt-sensitive rats fed with high salt diet, coronary arterial HO-1 immunostaining was enhanced, suggesting that coronary HO-1 expression was augumented, promoting increased coronary dilatation in salt-dependent hypertension [277].

The recombinant alpha1C subunit of the human cardiac L-type Ca^{2+} channel and L-type Ca^{2+} currents in rat cardiomyocytes can be inhibited by CO [283]. CO was also able to inhibit recombinant and native forms of this channel *via* ROS of mitochondrial origin [249,250].

Interestingly, H_2S, CO, GSH, and superoxide dismutase levels decreased, and malondialdehyde level increased during myocardial I/R in salt-sensitive rats treated with *hydroxylamine* and *zinc protoporphyrin*, a CBS inhibitor and a HO-1 inhibitor, respectively. Whereas HO-1-mRNA and CBS-mRNA levels decreased in comparison to rats subjected to I/R only, thus suggesting that both CBS/H_2S and HO-1/CO systems interact with each other and play a cardioprotective role in I/R [284].

On such basis, experimental CO releasing-molecules (CORMs) are being developed, in order to further study the cardioprotective potential of CO [285-288]. These compounds induce cardioprotection in both *ex vivo* and *in vivo* experiments, by a series of mechanisms that need to be further elucidated. Nevertheless, they seem to involve Akt [289], the cardiac stromal cell-derived factor-1α [290], inhibition of the mPTP [291], activation of the p38 MAPK β and PKC pathways before ischemia. Moreover, they may involve PI3K pathway during reperfusion [292] and activation of the KATP channels [293], Nrf2 and NF-κB, STAT1/3 [294]. Notably only physiologic concentrations of endogenous CO are cytoprotective, while excessive endogenous levels reflect underlying inflammation, oxidative stress and vascular pathology [264]. Furthermore, high levels of endogenous CO may lead to the production of ROS, [295] impairing NO mediated vasodilation, [296] and promoting adverse vascular remodeling [297]. Indeed, prolonged exposure to CO at high concentrations leads to deleterious effects on myocardial I/R injuries [298] and on cardiac remodeling and ventricular arrhythmias in rats [299,300].

Agents with unknown membrane target

Chromogranin A- derived peptides: Vasostatin-1 and Catestatin

Recent data have highlighted the role of two active endogenous peptides derived from *Chromogranin A* (CgA), *Vasostatin-1* (VS-1) and *Catestatin* (CST), in eliciting important protective effect on the heart undergoing I/R. CgA is a

48 kDa acidic glycoprotein of the granins family of proteins, expressed in secretory vesicles of neuroendocrine cells, neurons and other cells including cardiomyocytes, where is co-stored and co-secreted, respectively, with catecholamines and NPs. CgA is cleaved by cell and tissue-specific proteases to numerous peptides, some of them involved in the modulation of different physiological processes, in inflammatory reactions and in the innate immunity. Among these peptides, VS-1 and CST have been extensively studied as regulators of cardiovascular function, mainly because of their ability to counteract the adrenergic signal. Indeed, they are able to control catecholamine release from chromaffin cells and noradrenergic neurons, to exert *in vivo* and *in vitro* vasodilatory effects, and to limit the inotropic and lusitropic responses to β-adrenergic stimulation of the heart. The cardio-suppressive and vasodilator properties of VS-1 and CST have been recently explained as due to a PI3K-dependent-NO release by endothelial cells [301].

Interestingly, the initiating factor for this intracellular cascade has been shown to reside in the interaction with membrane proteoglycans, thus activating a PI3K-dependent caveolae endocytosis, as both peptides are cationic and amphipathic, and exhibit membrane-binding properties resembling those of the cell penetrating peptides [302,303].

Therefore, the established anti-adrenergic and endothelial PI3K/NO signaling of both VS-1 and CST have encouraged to also verify their cardioprotective features. At this regard Cappello *et al.* [304] showed that in the isolated rat heart exposed to I/R, VS-1 simulated PreC through two different pathways: the first one mediated by A1 receptors activation and the other by NO release. Moreover, VS-1 exerted protective effects on neonatal rat cardiomyocytes, both cultured alone and in the presence of aortic endothelial cells, suggesting the involvement of direct and endothelial dependent mechanisms [305,306].

In isolated rat hearts subjected to global ischemia CST, given for the first 20 min of reperfusion, exerted an infarct-sparing effect, reduced contracture and decreased the post-ischemic systolic dysfunction by preventing mPTP opening, with a mechanism involving PI3K/Akt and PKCs [307]. Conversely, in the isolated rat heart exposed to regional ischemia followed by reperfusion, wild type CST and the Pro370Leu variant (allele frequency ~0.3%) increased myocardial infarct size through Akt dephosphorylation [308]. These diverging results probably reside in the different experimental protocols (global *vs* regional ischemia) and CST dose applied, resulting in different patterns of tissue response to I/R.

Furthermore, CST was also found to be protective in a model of isolated ventricular myocytes subjected to simulated ischemia, suggesting the presence of a direct effect on cardiomyocytes, independent from the presence of catecholamine or of endothelial cells [307]. CST given in the early reperfusion reduces infarct size and improves cardiac function *via* a PI3K/Akt, PKC, mitochondrial KATP channels and ROS signaling in the postischemic non-hypertrophic and hypertrophic rat hearts, supporting a potential therapeutic role for CST, even in the presence of comorbidities, such as hypertension and cardiac hypertrophy [301,309].

Exosomes and microvesicles

Exosomes and *microvesicles/microparticles* are vesicles of 30-100 nm and 100-1000 nm in diameter, respectively, released by several cell types. They are collectively termed extracellular vesicles (EVs) and may represent a powerful tool of inter-cellular communication. In particular, exosomes are capable of transferring proteins, mRNA, and miRNA between cells and they can be proangiogenic and may have cardioprotective properties. In contrast, microvesicles, seem to have more frequently detrimental effects that are pro-thrombotic and pro-inflammatory.

Exosomes are released *via* an exocytic pathway from multivesicular bodies and have the potential for cell-specific targeting [310]. For instance, a conditioned medium derived from mesenchymal stem cell (MSC) has been shown to mediate cardioprotective effects during I/R protocols *via* large complexes of 50-100 nm. Therefore MSCs secretes 50- to 100-nm particles, which could be observed by electron microscopy. These particles are phospholipid vesicles composed of cholesterol, phosphatidylcholine and sphingomyelin as well as of exosome-associated proteins, *e.g.*, Alix, CD81 and CD9. These particles with a radius of 55-65 nm, purified as a homogeneous population of particles by size-exclusion fractionation on a HPLC, are likely exosomes. It has been shown that these "purified exosomes" reduced infarct size in a mice model of cardiac I/R injury. Hence, it was concluded that MSC has paracrine cardioprotective effect *via* exosomes secretion [311]. Recently, it has been reported that exosomes and a few more secreted membrane vesicles, *i.e.* EVs, act as paracrine signaling mediators within the heart. These vesicles derived from human cardiac progenitors cells [312] increased angiogenesis, decreased cardiomyocyte apoptosis, and improved LVEF. Also mouse cardiac progenitor-derived exosomes protected ischemic heart from acute I/R injury [313]. Exosomes derived from cardiosphere-derived cells have been also proposed as key mediators of cardiosphere-induced cardiac regeneration, [314]. Ischemic PreC markedly increased EV release from the heart. Administration of coronary perfusate from preconditioned hearts attenuated infarct size in non-preconditioned recipient hearts, similarly to cardioprotection afforded by PreC itself on the donor hearts. Perfusates of preconditioned hearts depleted of EVs failed to exert cardioprotection in recipient hearts. This has been the first demonstration that EVs released from the heart after ischemic PreC are necessary for cardioprotection by remote PreC, evidencing the importance of vesicular transfer mechanisms in remote cardioprotection [315]. It has also been suggested that exosomes present in plasma of humans and rats are protective in a Langendorff-perfused rat heart [316]. These studies highlight the importance of exosomes and vesicles in local and distant micro-communication mechanisms after myocardial infarction and their potential utility as cell-free therapeutic candidates. However caution must be used and extensive studies are necessary because the mechanisms of protection are still unknown.

Conclusions: the future of pharmacological conditioning

There are several endogenous cardioprotective factors (Table 1); we did not consider all of them for space constrain. Our analysis may provide a stimulus and useful heuristic approach for medical oriented research aiming to clarify the risk factors underlying the vulnerability of the human heart and the intrinsic properties of self-protection. Nevertheless, we must admit that there is no consistent evidence for cardioprotection by many endogenous protectant agents when used as pharmacological conditioning drugs in the clinical scenario of AMI, though they have been seen to be protective several times in animal models [46,47,105,317,318]. In addition to the potential confounding effects of comorbidities in man, usually absent in many animal models, the lack of evidence for cardioprotection of pharmacological or ischemic conditioning may relate to the fact that most widely used drugs in clinical scenario, are already cardioprotective, likely using the conditioning/protective RISK and SAFE pathways. Cardioprotective drugs which may "interfere" with conditioning include anxiolytics [319] and antiaggregants [45]. For example, ischemic PreC and PostC have been combined with the P2Y12 inhibitor *cangrelor* and no additive protection has been found [320]. Also anesthetics are cardioprotective [321,322] and this could be relevant in the surgical suite for cardiovascular and non-cardiovascular interventions with elevated risk of coronary accidents. ACE-I are drugs often used chronically for the treatment of patients with an elevated risk for coronary disease. With chronic use of ACE-I the incidence of AMI is reduced [323,324], and apart from more favorable hemodynamics, their antioxidant properties and pro-bradykinin, pro-nitric oxide effects may contribute to such benefit [240], thus limiting the possibility of additional protection by conditioning protocols [118]. Moreover statins, nitrates, and antidiabetic drugs may treat risk factors thus influencing cardioprotection by modifying cellular signaling involved in protection [231].

If it is true that drugs currently used in clinical practice interfere with conditioning and elicit the same pathways triggered by endogenous factors, future attempts to further decrease both the incidence and the mortality of myocardial infarction should be based on interventions which can prove protective *via* different mechanisms.

Therefore, future animal studies on cardioprotection should be conducted on top of standard therapy to have a more clinically relevant model. Interventions must demonstrate additive protection in such a model to be considered of clinical value. For instance, it deserves to be investigated whether interventions such as induction of autophagy or remote conditioning [325-327], whose mechanisms are less studied, might display additive effects in the presence of well-known cardioprotective therapies. As said, remote ischemic pre-, per- and post-conditioning can be obtained by repeated cycles of I/R in a limb by inflating/deflating a blood pressure cuff [15-18,46,250,315,325,328-333]. Remote conditioning resulted to be a powerful mean to reduce infarct size during programmed interventional and surgical coronary revascularization [328,330,332]. It has also been protective in patients with reperfused AMI [329] and may result protective in the long-term [331-333]. Therefore it may be worthwhile to test remote conditioning as a safe,

simple, inexpensive and effective cardioprotective approach in the presence of already cardioprotective therapies [334]. In this context the study of *exosomes* (see above) may open new perspectives.

It has been suggested that other cardioprotective interventions can be associated to enhance protection. For example, mild hypothermia [335,336] and the Na^+-H^+ exchanger inhibitor cariporide [337] result cardioprotective when applied during ischemia. Thus protection from reperfusion injury *via* PreC or PostC or substances given in reperfusion could be associated to cooling or cariporide which protect against ischemic damage. Recently it has been reported that the protective effect of the pharmacological PostC agent AMP579 [338] has been added to that of cariporide that protects against an ischemic injury [339]. Yet the addition of cangrelor and cariporide to mild hypothermia nearly abolished the I/R injury in a rat model [320]. Therefore, we must take into account the presence of comorbidities and associated therapies, which should or should not interfere with redox signaling and other cardioprotective pathways, when planning experiments for the validation and identification of cardioprotective drug targets and clinical studies [46,49,254].

By coupling the use of relevant animal models with comorbidities and with concomitant best therapies to further knowledge of endogenous cardioprotective pathways, we hope to identify appropriate candidates for each cardioprotective strategy to be tested in future clinical trials and one day defeat ischemic heart disease.

List of Abbreviations

3- MST = 3-mercaptopyruvate sulfurtransferase

AMI = Acute myocardial infarction

AMPK = AMP kinase

ANP = Atrial natriuretic peptide

AR = Adenosine receptor

BK = Bradykinin

BNP = Brain natriuretic peptide

CBS = Cystathionine beta-synthase

CgA = Chromogranin A

cGMP = Cyclic guanosine monophosphate

CHF = Chronic heart failure

CNP = C-type natriuretic peptide

CsA = Cyclosporine A

CSE = Cystathionine gamma-lyase

CST = Catestatin

CVD = Cardiovascular disease

CX43 = Connexin 43

DOR = δ-opioid receptor

EPCs = Endothelial progenitor cells

Epo = Erythropoietin

EpoR = Epo receptor

ERK = Extracellular signal-regulated kinase

EVs = Extracellular vesicles

GC = Guanylyl cyclase

GHRH = Growth hormone-releasing hormone

GHS = GH secretagogues

GHS-R = GHS receptor

GLP = Glucagon-like peptides

GPCRs = G protein-coupled receptors

GSK3β = Glycogen synthase kinase 3 beta

HIF-1 alpha = Hypoxia-inducible factor-1 alpha

HO = Heme oxygenases

I/R = Ischemia/reperfusion

JAK = Janus kinase

KOR = κ-opioid receptor ()

LV = Left ventricle

LVEF = LV ejection fraction

MAPK = Mitogen-activated protein kinase

MOR = μ-opioid

mPTP = Mitochondrial permeability transition pore

MSCs = Mesenchymal stem cells

NOS = Nitric oxide synthase

NPs = Natriuretic peptides

NRG1 = Neuregulin-1

PAF = Platelet activating factor

PAG = Propargylglycine

PerC = Perconditioning

PI3K = Phosphatidylinositol 3-kinase

PKB/Akt = Protein kinase B

PKC = Protein kinase C

PKG = Protein kinase G

PostC = Postconditioning

PreC = Preconditioning

RISK = Reperfusion Injury Salvage Kinase

RNS = Reactive nitrogen species

ROS = Reactive oxygen species

S1P = Sphingosine-1-phosphate

SAFE = Survivor Activating Factor Enhancement

SK = Sphingosine kinase

STAT3 = Signal transducer and activator of transcription 3

TNF-α = Tumor necrosis factor-alpha

TRAF2 = TNF receptor associated factor 2

VS-1 = Vasostatin-1

FIGURE LEGENDS

Figure 1. Ischemic conditioning consists of a series of brief periods of ischemia and performed before, during or after the infarcting/index ischemia, which elicit the release of several endogenous cardioprotective agents. These are depicted as circles, triangles and rectangles. Stars represent oxygen and nitrogen reactive species. Cardioprotective agents can be released by several cell types and may act in a paracrine/autocrine fashion to activate membrane receptors and to trigger intracellular pro-survival pathways. Cardioprotective pathways converge on mitochondria where they prevent mitochondrial permeability transition pore (mPTP) formation.

Figure 2. Specific protective actions of cardioprotective agents with metabolic effects.

Figure 3. The gasotransmitters nitric oxide (NO), hydrogen sulphide (H_2S) and carbon monoxide (CO), are produced by the action of enzymes and mediate cardioprotection acting on specific targets.

Conflict of Interest

None

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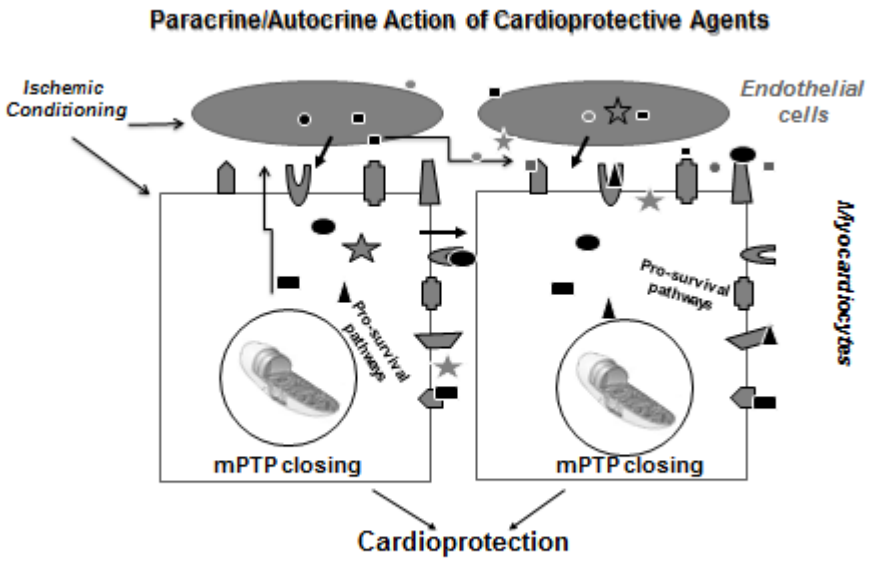

Paracrine/Autocrine Action of Cardioprotective Agents
Ischemic Conditioning
Endothelial cells
Myocardiocytes
Pro-survival pathways
Pro-survival pathways
mPTP closing
mPTP closing
Cardioprotection

Fig. 1

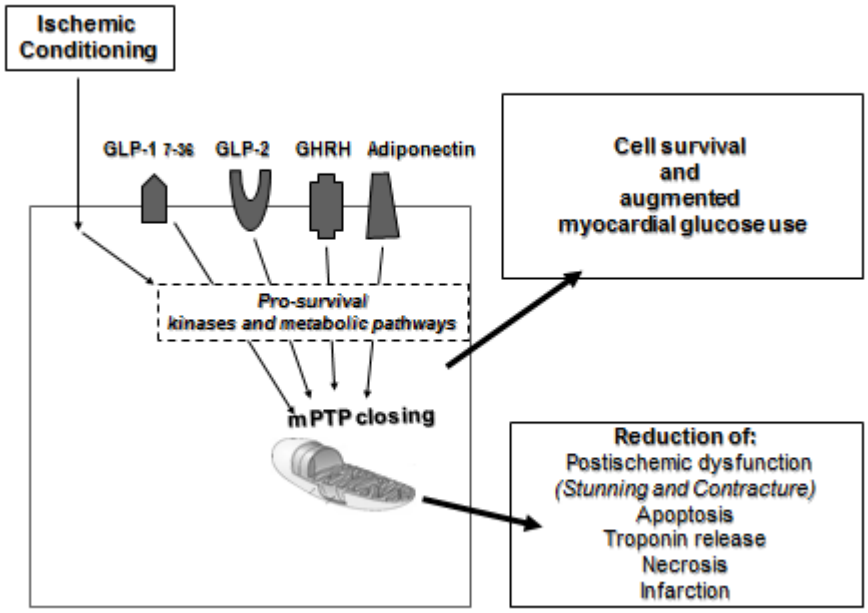

Ischemic Conditioning
GLP-1 7-36
GLP-2
GHRH
Adiponectin
Pro-survival kinases and metabolic pathways
mPTP closing
Cell survival and augmented myocardial glucose use
Reduction of:
Postischemic dysfunction
(Stunning and Contracture)
Apoptosis
Troponin release
Necrosis
Infarction

Fig. 2

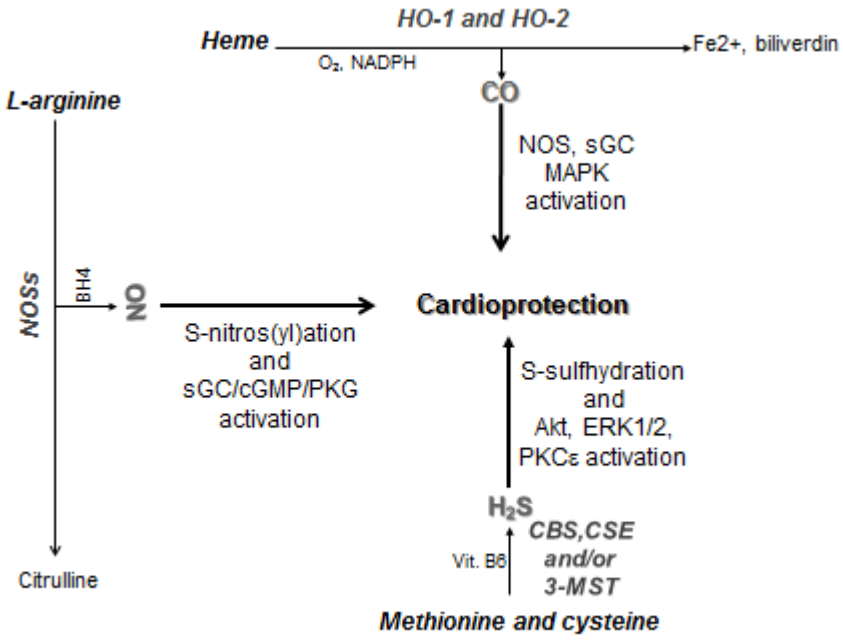
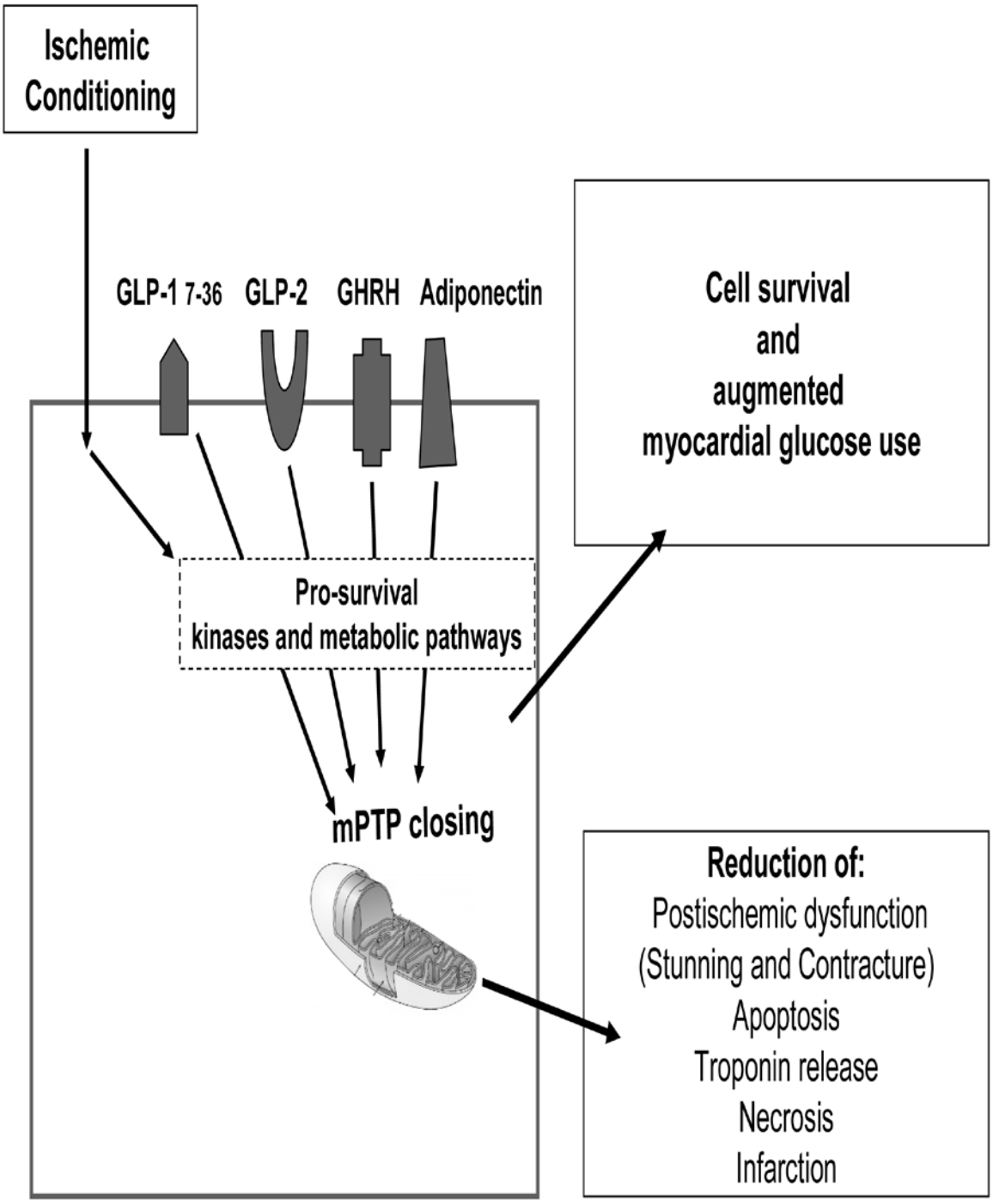

HO-1 and HO-2
Heme
O2, NADPH
Fe2+, biliverdin
CO
L-arginine
NOS, sGC
MAPK
activation
NOSs
BH4
NO
Cardioprotection
S-nitros(yl)ation
and
sGC/cGMP/PKG
activation
S-sulfhydration
and
Akt, ERK1/2,
PKCε activation
H2S
CBS,CSE
and/or
3-MST
Vit. B6
Citrulline
Methionine and cysteine

Fig. 3

Table 1. Endogenous cardioprotective factors

Substance	Time of Administration	I/R Injury	Clinical trials	Ref
Adenosine	Pre and Post	↓	Yes	80-87,92,93
Adipocytokines	Pre and Post	↓	None	101,102,108,109
Bradykinin	Pre and Post	↓	Yes	111-114,121,122
Glucagon-like peptide	Pre and Post	↓	Yes	123,125,126,136
Opiods	Pre and Post	↓	None	84,150-153
PAF	Pre	↓	None	52,160-162
GHRH	Pre and Post	↓	None	166-168
EPO	Pre	↓	Yes	171,174-177
TPO	Pre and Post	↓	None	189,190
Insulin	Pre	↓	None	199-201
NRG	Pre and Post	↓	Yes	210,218-220
Ghrelin-associated peptides	Pre and Post	↓	None	224,232,233
Natriuretic peptides	Pre and Post	↓	Yes	75,240-243,247
Gasotransmitters	Pre and Post	↓	Yes	9,250,253-255,259,260,268-274,277,278,281,282
Chromogranin A-derived peptides	Pre and Post	↓	None	304,307,309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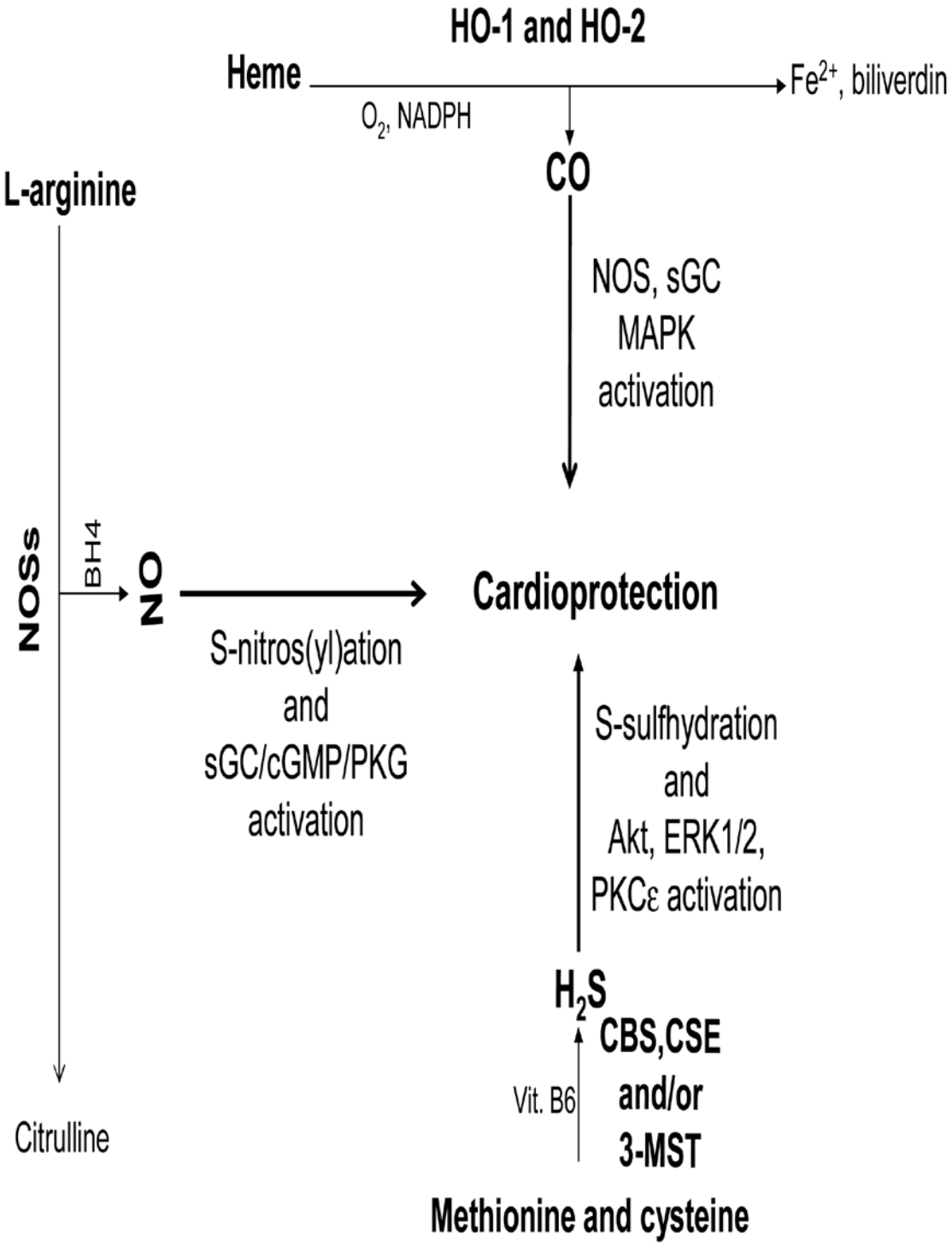
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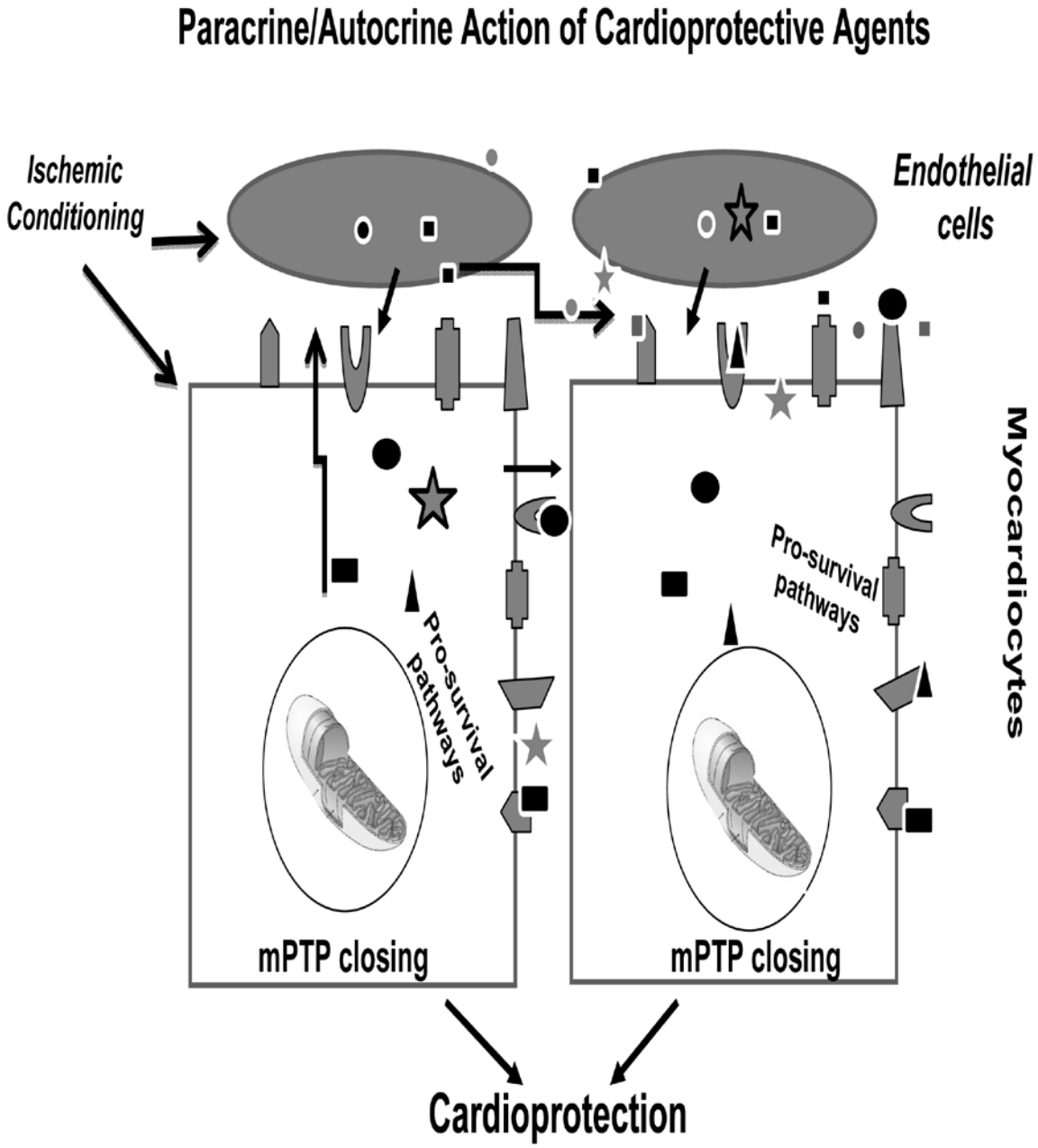
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